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Chemical and Biological Reduction of the Radical SAM Enzyme CPH_4 Synthase

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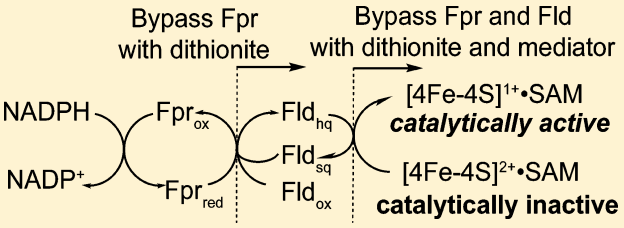
Supporting Information

ABSTRACT: The radical *S*-adenosyl-L-methionine (SAM) superfamily is a large and growing group of enzymes that conduct complex radical-mediated transformations. A one-electron reduction of SAM via the +1 state of the cubane [4Fe-4S] cluster generates a 5′-deoxyadenosyl radical, which initiates turnover. The [4Fe-4S] cluster must be reduced from its resting +2 state to the catalytically active +1 oxidation state by an electron. In practice, dithionite or the *Escherichia coli* flavodoxin (*Ec*FldA)/ferredoxin (flavodoxin):$NADP^+$ oxidoreductase (Fpr)/NADPH system is used. Herein, we present a systematic investigation of the reductive activation of the radical SAM enzyme CDG synthase (*Bs*QueE) from *Bacillus subtilis* comparing biological and chemical reductants. These data show that either of the flavodoxin homologues encoded by the *B. subtilis* genome, *Bs*YkuN or *Bs*YkuP, as well as a series of small molecule redox mediators, supports *Bs*QueE activity. With dithionite as a reductant, the activity of *Bs*QueE is ∼75-fold greater in the presence of *Bs*YkuN and *Bs*YkuP compared to that in the presence of dithionite alone. By contrast, *Ec*FldA supports turnover to ∼10-fold greater levels than dithionite alone under the same conditions. Comparing the ratio of the rate of turnover to the apparent binding constant for the flavodoxin homologues reveals 10- and 240-fold preferences for *Bs*YkuN over *Bs*YkuP and *Ec*FldA, respectively. The differential activation of the enzyme cannot be explained by the abortive cleavage of SAM. We conclude from these observations that the differential activation of *Bs*QueE by Fld homologues may reside in the details of the interaction between the flavodoxin and the radical SAM enzyme.



The radical *S*-adenosyl-L-methionine (SAM) superfamily is a rapidly expanding group of enzymes that catalyze a myriad of biological transformations in primary and secondary metabolism, including cofactor biosynthesis/assembly and RNA modifications. Despite the rich functional diversity, the majority of the members of the radical SAM superfamily engage the three cysteine thiolates in the conserved CX_3CX_2C motif to coordinate three irons of a catalytically essential [4Fe-4S] cluster.[1] The fourth iron is coordinated by the α-carboxylate and α-amino moieties of the SAM. In nearly all radical SAM enzymes described to date, the +1 state of the [4Fe-4S] cluster supplies one electron to SAM generating a 5′-deoxyadenosyl radical (dAdo•) and L-methionine[2,3] (Scheme 1).

Reduction of the [4Fe-4S] cluster to the +1 oxidation state from the resting +2 form is common to all members of the radical SAM superfamily. This one-electron reduction requirement was first reported for pyruvate formate-lyase activating enzyme and lysine 2,3-aminomutase nearly 30 years prior to the unification of the radical SAM superfamily.[4,5] Although the biological reductant has never been directly identified, Knappe and co-workers determined that pyruvate formate-lyase activating enzyme isolated from *Escherichia coli* was reduced by *E. coli* flavodoxin (*Ec*FldA), suggesting a role for this ubiquitous protein in the reductive activation process.[4,6] In addition, *Ec*FldA reductively activates *E. coli* anaerobic ribonucleotide reductase and biotin synthase by supplying reducing equivalents from NADPH via ferredoxin (flavodoxin):$NADP^+$ reductase (Fpr).[7−10] Subsequent studies of additional radical SAM enzymes from *E. coli* with *Ec*FldA/Fpr and NADPH showed this to be a general feature of these proteins.[11,12] Interestingly, Brazeau et al. identified that *Ec*FldA and Fpr were capable of reductively activating lysine 2,3-aminomutase from *Porphyromonas gingivalis*, demonstrating that the *Ec*FldA and Fpr proteins could reductively activate a radical SAM enzyme from a different species.[13] Though *Ec*FldA and Fpr reductively activate radical SAM enzymes from diverse organisms, there has yet to be a systematic study of reductive activation. The closest systematic investigation comparing dithionite and *Ec*FldA/Fpr was reported in two studies by Booker and co-workers on the anaerobic sulfatase maturating enzymes, AtsB and anSMEcpe. The $V_{max}/[E_T]$ for each enzyme was 10−100-fold greater when dithionite was used as the reductant than when the *E. coli* biological system was used.[14,15] This observation underscores the need to more closely examine interactions between radical SAM enzymes and *in vivo* reducing systems.

7-Carboxy-7-deazaguanine (CDG) synthase (QueE) is a member of the radical SAM superfamily that catalyzes the radical-mediated ring contraction of 6-carboxy-5,6,7,8-tetrahy-

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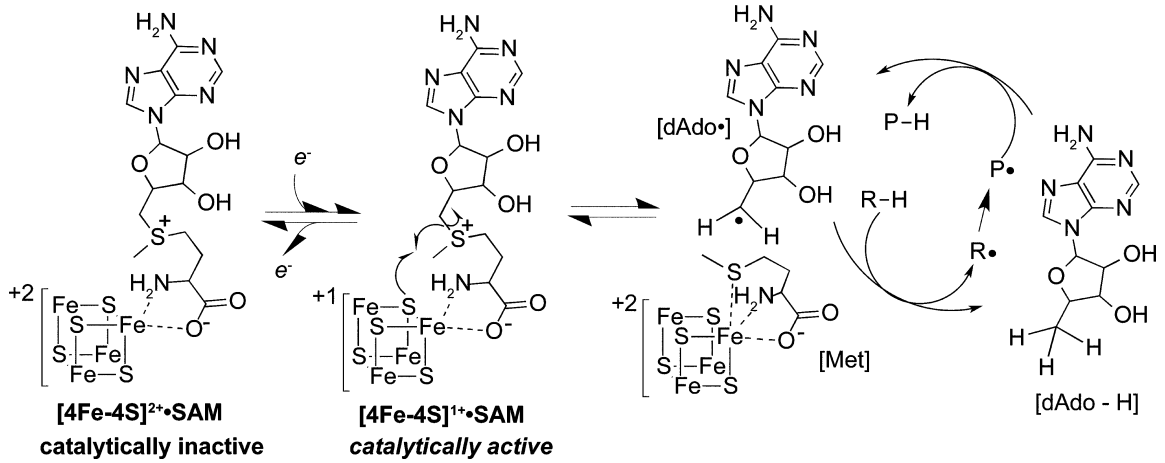
Scheme 1. Reductive Cleavage of SAM



dropterin (CPH_4) to form CDG.[16] Recent investigations of QueE homologues from *Burkholderia multivorans* and *Bacillus subtilis* have yielded rich structural and functional insights into the mechanism of ring contraction.[17,18] Additionally, the fact that both dithionite and the *E. coli* biological reducing system can supply reducing equivalents to *B. subtilis* QueE (*Bs*QueE) *in vitro* provides an ideal baseline to systematically investigate reductive activation.[17,18] In this paper, we compare the activity of *Bs*QueE in the presence of various chemical and biological reductants, highlighting the importance of the reducing system in studying radical SAM enzymes.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Protein Purification. His_6-tagged *Bs*QueE and the His_6-tagged *E. coli* flavodoxin reductase (Fpr) used in this investigation were cloned, expressed, and purified as previously described.[16,17] The genes *ykuN* and *ykuP*, which encode the two flavodoxin homologues from *B. subtilis* subsp. *subtilis* str. 168, were amplified from genomic DNA via polymerase chain reaction (PCR) using primers (Table 1 of the Supporting Information) that incorporated *Nde*I and *Xho*I for *ykuN* or *Nhe*I and *Xho*I for *ykuP* at the 5′- and 3′-ends of each gene, respectively. The *fldA* gene was amplified from *E. coli* W3110 genomic DNA via PCR using primers (Table 1 of the Supporting Information) that incorporated *Nde*I and *Eco*RI restriction sites at the 5′- and 3′-ends of the gene, respectively. The purified PCR products were A-tailed and ligated into pGEM Easy-T vector (Promega) for blue-white screening and sequencing. The corresponding inserts were digested from the pGEM Easy-T vector with *Nde*I/*Xho*I (*ykuN*) or *Nde*I/*Eco*RI (*fldA*) and ligated into pET29a digested in with the same enzymes. The *ykuP* gene was digested from the pGEM vector with *Nhe*I/*Xho*I and ligated into similarly digested pET21b (Novagen). Sequencing confirmed the identity of each construct prior to overexpression.

BL21(DE3) cells (Novagen) containing the *ykuN*-pET29a or *fldA*-pET29a constructs were grown in LB supplemented with 34 μg/mL kanamycin at 37 °C. HMS-174 (DE3) cells containing the *ykuP*-pET21b construct were grown in LB supplemented with 100 μg/mL ampicillin at 37 °C. Protein expression was induced via the addition of IPTG to a final concentration of 0.1 mM at an OD_{600} of ∼0.5. The medium was also supplemented with 0.1 mM riboflavin (final concentration) at the time of induction. Cells were harvested after ∼18 h, flash-frozen in liquid nitrogen, and stored at −80 °C until they were purified.

All chromatography was conducted at 4 °C. Frozen cells (1 g) were suspended in ∼3 mL of lysis buffer containing 50 mM Tris-HCl (pH 7.2), 10 mM DTT, and 1 mM EDTA. PMSF was added to a final concentration of 1 mM. Cells were lysed using a Branson digital sonifier operated at 50% amplitude. The lysate was centrifuged at 18000*g* for 35 min, and the supernatant was loaded onto a DEAE-Sepharose column (2.6 cm × 12.5 cm, GE Lifesciences) equilibrated in lysis buffer. The column was washed with lysis buffer, and protein was eluted using a linear gradient from 0 to 1 M KCl in the same buffer. Fractions containing flavodoxin were identified via SDS−PAGE analysis, pooled, and dialyzed overnight against buffer containing 50 mM HEPES-NaOH (pH 7.4), 0.1 M KCl, and 10 mM DTT. At this stage, *Ec*FldA was >95% pure so it was concentrated, and aliquots were flash-frozen in liquid nitrogen.

*Bs*YkuN and *Bs*YkuP were further purified using hydrophobic interaction chromatography as follows. Fractions containing *Bs*YkuN were pooled and prepared for hydrophobic interaction chromatography by adding solid ammonium sulfate to a final concentration of 1.5 M over 1 h. The solution was loaded onto a butyl sepharose column (2.6 cm × 12.5 cm, GE Lifesciences) equilibrated in 50 mM Tris-HCl (pH 7.2), 2 mM DTT, 1 mM EDTA, and 1.5 M ammonium sulfate. The column was washed with loading buffer, and the protein was eluted with a linear gradient from 1.5 to 0 M ammonium sulfate. Fractions containing *Bs*YkuN were pooled and dialyzed overnight at 4 °C against 20 mM HEPES-NaOH (pH 7.5) containing 0.1 M KCl and 2 mM DTT. *Bs*YkuP was purified essentially as described above except the ammonium sulfate concentration was 2 M and the column was equilibrated in buffer containing 2 M ammonium sulfate and eluted with a linear gradient from 2 to 0 M.

*Bs*YkuN and *Bs*YkuP were concentrated to a minimal volume, and 1 mL fractions were injected onto a HiPrep 16/60 Sephacryl S200 high-resolution size exclusion column (GE Lifesciences) for further purification. The column was equilibrated with dialysis buffer, and the flavodoxins were eluted isocratically running at 0.5 mL/min. Fractions containing the proteins were pooled on the basis of color and SDS−PAGE. Each flavodoxin was concentrated to a minimal volume and flash-frozen in liquid nitrogen.

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*Ec*FldA, *Bs*YkuN, and *Bs*YkuP were quantified on the basis of A_{467} and using extinction coefficients (ε_{467}) of 8250 M^{-1} cm^{-1} (*Ec*FldA), 10700 M^{-1} cm^{-1} (*Bs*YkuN), and 9930 M^{-1} cm^{-1} (*Bs*YkuP).[19] The extinction coefficients of *Bs*YkuN and *Bs*YkuP were experimentally determined in triplicate as follows. *Bs*YkuN and *Bs*YkuP were diluted separately into 0.1 M potassium phosphate (pH 7.3) to four different concentrations such that the measured absorbance of each dilution was between 0.1 and 0.8 at 467 nm. The UV−visible spectrum of each sample was measured on an Agilent 8453 diode array spectrophotometer. Next, aliquots (135 μL) of each dilution were treated with 15 μL of 30% (w/v) TCA to acidify the solution and boiled for 10 min to denature the flavodoxin and release the FMN cofactor. The boiled samples were centrifuged at 17000*g* for 10 min to remove the precipitated protein. The absorbance of the supernatant was measured at 445 nm to determine the amount of free FMN in solution. The concentration of free FMN was calculated using an ε_{445} of 12500 M^{-1} cm^{-1}, factoring in the dilution due to the addition of TCA. The FMN concentration measured after the addition of TCA and boiling is equivalent to the *Bs*YkuN or *Bs*YkuP concentration with bound FMN. The absorbance measured at 467 nm from *Bs*YkuN or *Bs*YkuP in 0.1 M potassium phosphate (pH 7.3) was plotted versus the calculated *Bs*YkuN or *Bs*YkuP concentration to calculate the molar extinction coefficient of the flavodoxins. FMN from Sigma was diluted in 0.1 M potassium phosphate (pH 7.3) and acid treated and boiled in the same manner to ensure that the extinction coefficient of FMN at 445 nm does not change during the acid treatment and boiling.

Synthesis of Substrates. SAM and CPH_4 were enzymatically synthesized as described previously.[17]

Time Course Using Dithionite as a Reductant. All kinetic experiments described below monitor the turnover of *Bs*QueE by measuring the rate of CDG formation in triplicate. The reaction mixtures were set up under anaerobic conditions (95% N_2/5% H_2 atmosphere in a Coy anaerobic chamber) and contained 50 mM PIPES-NaOH (pH 7.4), 10 mM DTT, 2 mM $MgSO_4$, 2 mM SAM, 2 mM CPH_4, 2 μM *Bs*QueE, and 10 mM dithionite. All components except CPH_4 were mixed and incubated at room temperature for 10 min. Turnover was initiated upon addition of CPH_4. Aliquots (45 μL) were withdrawn at various times and quenched by mixing with 4.5 μL of 30% (w/v) TCA.

Aliquots of the reaction mixture were analyzed by HPLC as follows. A sample from each reaction mixture was injected onto an Eclipse XDB-C18 reverse phase column (Agilent) and eluted with a linear gradient from 0 to 10% acetonitrile with 0.1% (v/v) TFA. The peak corresponding to CDG was integrated and quantified by comparison with peaks obtained at known concentrations of a CDG standard.

Time Course with a Biological Reducing System. The reactions with the biological reducing system were conducted as described above for dithionite with minor modifications as follows. Reaction mixtures contained 50 mM PIPES-NaOH (pH 7.4), 10 mM DTT, 2 mM $MgSO_4$, 2 mM SAM, 2 mM CPH_4, 1 μM *Bs*QueE, 2 mM NADPH, and either 2 μM Fpr and 5 μM *Bs*YkuN, 5 μM Fpr and 20 μM *Bs*YkuP, or 5 μM Fpr and 20 μM *Ec*FldA. All components except CPH_4 were mixed and incubated at room temperature for 10 min. The reaction was initiated upon addition of CPH_4. Aliquots (60 μL) were withdrawn at various times and quenched by mixing with 6 μL of 30% (w/v) TCA. CDG was quantified as described above. Negative controls for each flavodoxin were run in the same manner except either NADPH or Fpr was omitted from each reaction.

$$v = \left\{ V_{max}(([QueE]_t + [Fld]_t + K_{Fld}) - \sqrt{([QueE]_t + [Fld]_t + K_{Fld})^2 - 4[QueE]_t[Fld]_t} \right\} / \left(2[QueE]_t\right) \quad (1)$$

Flavodoxin Dependence of *Bs*QueE Activity with Fpr/NADPH as a Reductant. Reaction mixtures contained 50 mM PIPES-NaOH (pH 7.4), 10 mM DTT, 2 mM $MgSO_4$, 2 mM SAM, 2 mM CPH_4, 1 μM *Bs*QueE, and 2 mM NADPH. The concentrations of Fpr (1 or 5 μM for *Bs*YkuN and 5 or 20 μM for *Bs*YkuP and *Ec*FldA) and each flavodoxin (0.39, 0.77, 3.9, 7.7, or 23.1 μM for *Bs*YkuN, 0.42, 0.83, 4.2, 8.3, or 24.9 μM for *Bs*YkuP, and 1, 5, 10, 20, and 40 μM for *Ec*FldA) were varied. All components except CPH_4 were mixed and incubated at room temperature for 10 min. The reaction was initiated upon addition of CPH_4. Each reaction mixture was set up with a final volume of 60 μL and each reaction quenched 1 min after addition of CPH_4 by mixing with 6 μL of 30% (w/v) TCA. An aliquot of each reaction mixture was analyzed by HPLC for CDG as described above.

The initial rate of formation of CDG as a function of Fld concentration was fit to eq 1. The V_{max} (k_{CDG}) and K_{Fld} values from several data sets were averaged to obtain the reported standard deviations.

Flavodoxin Dependence of *Bs*QueE Activity with Dithionite as a Reductant. The reactions with flavodoxin using dithionite as a reductant were conducted as described above with minor modifications as follows. Reaction mixtures contained 50 mM PIPES-NaOH (pH 7.4), 10 mM DTT, 2 mM $MgSO_4$, 2 mM SAM, 2 mM CPH_4, 1−2 μM *Bs*QueE, 10 mM dithionite, and various concentrations (0−30 μM) of flavodoxin. All components except CPH_4 were mixed and incubated at room temperature for 10 min. The reaction was initiated upon addition of CPH_4. Each reaction mixture was set up with a final volume of 60 μL and each reaction quenched 1 min after addition of CPH_4 by mixing with 6 μL of 30% (w/v) TCA. CDG was quantified as described above.

Methyl Viologen Dependence of *Bs*QueE Activity with Dithionite as a Reductant. To examine if methyl viologen could replace flavodoxin as a mediator in the reaction, *Bs*QueE activity was assayed as follows. Reaction mixtures contained 50 mM PIPES-NaOH (pH 7.4), 10 mM DTT, 2 mM $MgSO_4$, 2 mM SAM, 2 mM CPH_4, 1−2 μM *Bs*QueE, 10 mM dithionite, and 0−10 mM methyl viologen. All components except CPH_4 were mixed and incubated at room temperature for 10 min. The reaction was initiated upon addition of CPH_4. Each reaction mixture was set up with a final volume of 60 μL and each reaction quenched 1 min after addition of CPH_4 by mixing with 6 μL of 30% (w/v) TCA. CDG was quantified as described above. The initial rate of CDG formation as a function of methyl viologen concentration was fit to eq 2 to obtain the V_{max} (k_{CDG}) value from triplicate data sets, which was averaged to obtain the reported standard deviation.

$$v = \frac{V_{max}[\text{methyl viologen}]}{K + [\text{methyl viologen}]} \quad (2)$$

Redox Mediator Dependence of 5′-Deoxyadenosine and CDG Formation. Reaction mixtures contained 50 mM

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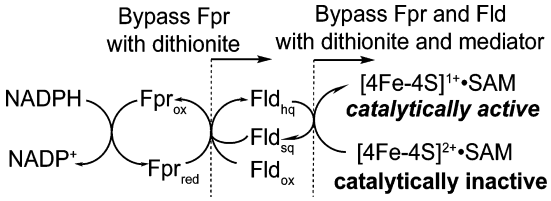
Biochemistry 2015, 54, 2903−2910

PIPES-NaOH (pH 7.4), 10 mM DTT, 2 mM $MgSO_4$, 2 mM SAM, 2 mM CPH_4, 2 μM *Bs*QueE, 10 mM dithionite, and either no mediator, 2 mM methyl viologen (E_m = −0.446 V),[20] 2 mM benzyl viologen (E_m = −0.359 V),[20] 1 mM neutral red (E_m = −0.325 V),[21] or 1 mM lissamine blue BF (E_m = −0.276 V, at pH 7.5).[22] All components except CPH_4 were mixed and incubated at room temperature for 10 min. The reaction was initiated by addition of CPH_4. Aliquots (65 μL) from the 420 μL total reaction mixture were withdrawn at 0.25, 0.5, 0.75, 1, 1.5, and 2 min and quenched by mixing with 6.5 μL of 30% (w/v) TCA. The 5′-deoxyadenosine formed was quantified with the same HPLC separation method described above for CDG.

RESULTS

All members of the radical SAM superfamily require the one-electron reduction of the $[4Fe\text{-}4S]^{2+}$ cluster to the +1 oxidation state. This can be achieved by using a chemical reductant, such as dithionite, or a biological reducing system in which the reducing equivalents are derived from NADPH and transferred to the cluster via flavodoxin reductase and flavodoxin (Scheme 2). Previous investigations of *Bs*QueE showed that reducing equivalents could be derived from either dithionite or NADPH via the *E. coli* Fpr/FldA reducing system, with the biological system acting as the better reductant.[17] While the *E. coli* Fpr/FldA/NADPH system is capable of reductively activating *Bs*QueE, not all members of the radical SAM superfamily can be activated by this system. The fact that *Bs*QueE is reductively activated by both dithionite and the *E. coli* biological reducing system affords an opportunity to further investigate the reductive activation step of a radical SAM enzyme.

Scheme 2. Electron Flow for [4Fe-4S] Cluster Reduction[a]



[a]The scheme shows the flow of electrons and does not imply absolute stoichiometry.

Activation of CDG Synthase: Biological versus Chemical Reduction. While dithionite and the *E. coli* FldA/Fpr/NADPH system activated *Bs*QueE, the *in vivo* reductant for this enzyme has yet to be determined. The *B. subtilis* genome contains two *orfs*, *ykuN* and *ykuP*, that encode homologues of the *Ec*FldA. We hypothesized that either or both of these enzymes may support reductive activation of *Bs*QueE, where the reducing equivalents would be derived from NADPH via Fpr.[23] To test this hypothesis, the genes *ykuN* and *ykuP* were cloned, the proteins encoded by each gene were heterologously expressed in *E. coli*, and *Bs*YkuN and *Bs*YkuP were purified to homogeneity. Initial experiments tested multiple conditions for reductive activation: dithionite alone, NADPH/Fpr/*Ec*FldA, NADPH/Fpr/*Bs*YkuN, and NADPH/Fpr/*Bs*YkuP (Figure 1); in these experiments, the *E. coli* Fpr was used to couple NADPH oxidation to cluster reduction.[24] The results show that although *Bs*QueE is active with dithionite (0.2 min^{-1}), 75-fold higher rates are observed in assays containing the *B. subtilis* Fld homologues. Moreover, the *Bs*Fld homologues support turnover 10-fold more than *Ec*FldA does.

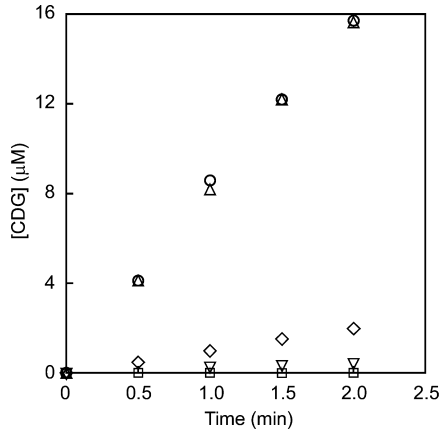


Figure 1. Time course of *Bs*QueE reaction under various reducing conditions. Activity was monitored by measuring CDG formation. *Bs*QueE activity was measured in the presence 10 mM dithionite (▽) or 2 μM Fpr and 2 mM NADPH with 5 μM *Bs*YkuN (○), 5 μM Fpr and 2 mM NADPH with 20 μM *Bs*YkuP (△), or 5 μM Fpr and 2 mM NADPH with 20 μM *Ec*FldA (◇). Assays in which Fpr or NADPH (□) was omitted served as negative controls.

Control experiments in which either NADPH or Fpr was omitted exhibited no detectable activation of *Bs*QueE. Collectively, these results support the notion depicted in Scheme 2 that flavodoxin mediates electron transfer to *Bs*QueE and highlight a differential capacity for Fld homologues to support QueE activity.

Investigation of the Flavodoxin-Dependent Activation of *Bs*QueE. The simplest interpretation of the differential activation of *Bs*QueE by *E. coli* and *B. subtilis* Fld homologues shown in Figure 1 is that the *Bs*Fld homologues are optimized for activation of the cognate radical SAM enzyme. However, there are two factors, the Fld·*Bs*QueE protein−protein interaction and the reduction potentials of the FMN cofactor of Fld and the $[4Fe\text{-}4S]^{2+}$ cluster of *Bs*QueE, that can contribute to the flavodoxin-dependent reductive activation. There are only limited data in the literature about the binding of flavodoxin to a protein. In methionine synthase, Matthews and co-workers demonstrated by NMR and cross-linking that flavodoxin interacts with the activation module of the protein.[25,26]

To probe the selectivity of Fld homologues in supporting turnover by *Bs*QueE, we examined the concentration dependence of activation at various Fld/Fpr levels. We reasoned that at low concentrations of Fld the Fld·*Bs*QueE interaction will dominate reductive activation, while at saturating concentrations of Fld, the reduction potentials will be limiting. For the sake of simplicity, the concentration of Fpr was kept constant at two concentrations (4−5-fold apart), which were both saturating, to isolate the Fld dependence of *Bs*QueE activity. Very similar saturation profiles were obtained for each Fld homologue at both Fpr concentrations (Figure 2A). The Fld concentration dependence exhibits saturation kinetics and was analyzed accordingly. The fits to the experimental data are summarized in Table 1. Both of the *B. subtilis* Fld homologues are more effective in supporting turnover by *Bs*QueE than the *Ec*FldA. Moreover, *Bs*YkuN exhibits a K_{Fld} for binding to *Bs*QueE that is substantially lower than those of *Bs*YkuP and *Ec*FldA. The data with *Bs*YkuN are not fit as well as those with *Ec*FldA and *Bs*YkuP because the lowest concentration of *Bs*YkuN employed (~0.4 μM) was clearly above the level of

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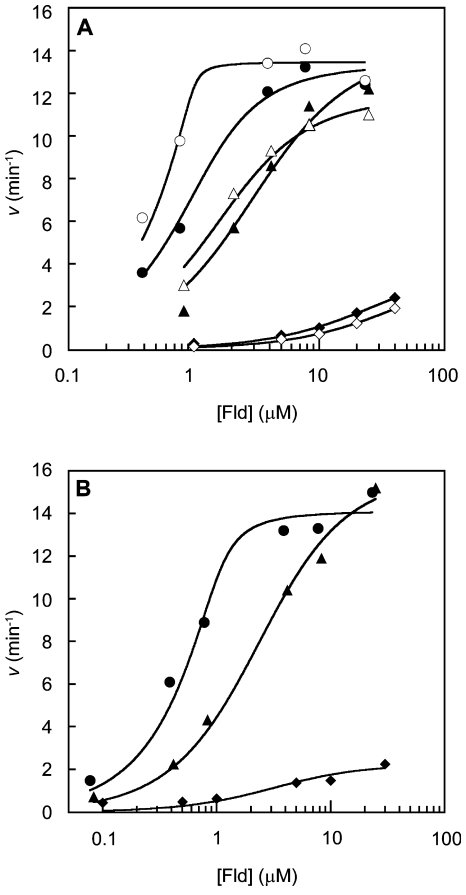

Figure 2. *Bs*QueE activity is enhanced by increasing the flavodoxin homologue [*Ec*FldA (diamonds), *Bs*YkuN (circles), or *Bs*YkuP (triangles)] concentration when the electrons are supplied by either NADPH via Fpr (A) or dithionite (B). To confirm Fld reduction by NADPH/Fpr was saturating, the rate of CDG formation depending on Fld concentration was measured at 1 μM (filled symbols) and 5 μM (empty symbols) Fpr for *Bs*YkuN and 5 μM (filled symbols) and 20 μM (empty symbols) for *Bs*YkuP and *Ec*FldA (A). Depicted are representative data sets for each experiment. The data were fit using eq 1, and the V_{max} (k_{CDG}) and K_{Fld} values are listed in Table 1.

half-maximal saturation. Nevertheless, we would conservatively place an upper limit of K_{BsYkuN} at <0.4 μM. The k_{CDG}/K_{Fld} ratio obtained by fitting the data for each Fld homologue provides a measure of efficiency of Fld for activating *Bs*QueE (Table 1). The data show that *Bs*YkuN is the most efficient activator of *Bs*QueE by at least 10-fold relative to *Bs*YkuP and >200-fold relative to *Ec*FldA, perhaps indicating the absence of optimized interaction surfaces between the *E. coli* flavodoxin and *Bs*QueE.

While the biologically relevant reducing system supports *Bs*QueE activity the best, the electron transfer system is complex, requiring three separate redox events, reduction of Fpr, electron transfer to Fld, and electron transfer to QueE, and for the Fld to interact with two different proteins, presumably in a mutually exclusive manner (Scheme 2). Furthermore, the flavodoxin reductase used in these studies was from *E. coli*, and in some of the experiments, the Fpr protein was present in up to 40-fold molar excess over flavodoxin. Thus, we were concerned that the differences in the activation profiles shown in Figure 2A may reflect mismatches between the Fpr−Fld interaction surfaces, rather than differences between the Fld−*Bs*QueE interaction.

To simplify the reduction system, dithionite was used as the reductant to bypass the Fpr/NADPH requirement (Scheme 2). Dithionite alone reductively activated *Bs*QueE, but it was a poor reductant with a k_{CDG} of 0.2 min^{-1}. Therefore, in experiments conducted in the presence of Fld homologues, any increase in *Bs*QueE activity would be solely due to the presence of the Fld. Indeed, addition of Fld to the *Bs*QueE reaction mixtures containing dithionite stimulated the rate of CDG formation in an Fld concentration-dependent manner (Figure 2B). As in Figure 2A, the data in Figure 2B display saturation kinetics revealing that Fpr is dispensable for *Bs*QueE activation and that dithionite is capable of delivering reducing equivalents to *Bs*QueE via Fld *in vitro*. The apparent binding (K_{Fld}) and maximal activity (k_{CDG}) parameters were obtained by fitting the data in Figure 2B. The maximal activities achieved with the *B. subtilis* Fld homologues (Table 1) were comparable when either Fpr/NADPH or dithionite was used as the electron source (12−15 and 15−16 min^{-1} with *Bs*YkuN and *Bs*YkuP, respectively) (Table 1). The *E. coli* flavodoxin (*Ec*FldA) supported activity 9-fold greater than with dithionite alone.

The large differences in activation by Fld proteins from different sources may arise from either the interaction between Fld and *Bs*QueE, the midpoint potentials of the FMN cofactors in Fld, or both. The data in panels A and B of Figure 2 show distinct differences in the K_{Fld} parameter, which may be interpreted as approximating the constant for binding of Fld to *Bs*QueE. The midpoint potentials for *Bs*YkuN and *Bs*YkuP are very similar (−0.382 and −0.378 V, respectively, for the hydroquinone/semiquinone one-electron couple), and both are approximately 0.05 V more positive than the midpoint reduction potential for *Ec*FldA (−0.433 V for the hydroquinone/semiquinone one-electron couple).[23,24] However, the attribution of the differential activation to one of these factors is complicated. *Bs*YkuP and *Bs*YkuN show an at least 10-fold difference in the measured K_{Fld} values; however, the maximal

Table 1. *Bs*QueE Kinetic Parameters Determined Using Various Reduction Conditions

	*Ec*FldA			*Bs*YkuN			*Bs*YkuP		
	dithionite	5 μM Fpr	20 μM Fpr	dithionite	1 μM Fpr	5 μM Fpr	dithionite	5 μM Fpr	20 μM Fpr
K_{Fld} (μM)	3.9 ± 1.4	29.0 ± 3.0	42.0 ± 9.0	0.13 ± 0.05[a]	0.3 ± 0.1[a]	0.09 ± 0.03[a]	2.0 ± 0.2	2.1 ± 0.2	1.1 ± 0.1
k_{CDG} (min^{-1})	1.8 ± 0.5	4.2 ± 0.7	4.9 ± 0.8	14.6 ± 1.0	12.4 ± 1.1	12.4 ± 1.1	16.5 ± 0.7	10.7 ± 3.4	9.7 ± 2.7
k_{dAdo} (min^{-1})	0.28	nd[c]	nd[c]	0.26	nd[c]	nd[c]	0.26	nd[c]	nd[c]
k_{CDG}/K_{Fld} (μM^{-1} min^{-1})	0.46 ± 0.21	0.14 ± 0.03	0.12 ± 0.03	110 ± 40[b]	41 ± 14[b]	140 ± 50[b]	8.3 ± 1.9	5.1 ± 1.7	8.8 ± 2.6
k_{CDG}/K_{dAdo}	6.4	nd[c]	nd[c]	56	nd[c]	nd[c]	64	nd[c]	nd[c]

[a]These values should be considered a lower limit because at the lowest concentration of *Bs*YkuN significant activity was observed. [b]Given the uncertainties in the K_{Fld} value, these should be considered lower limits. [c]Not detectable.

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Biochemistry 2015, 54, 2903−2910

activities observed by these Flds are the same, and both are higher than that observed with *Ec*FldA.

The data presented up to this point suggest that the differences in the ability of Fld homologues to support turnover may result from differences in interactions between Fld and *Bs*QueE. However, as we note above, there are differences between the published midpoint potentials of the Fld homologues used. Therefore, we examined if there is a correlation between the ability to support turnover and the midpoint potential using chemical mediators. Preliminary experiments with methyl viologen (E_m = −0.446 V) indicated that *Bs*QueE could be activated to the same levels (k_{CDG} = 13.6 ± 3.8 min^{-1}) as is achieved by saturating concentrations of *Bs*Fld with dithionite or Fpr/NADPH (Figure 3). We

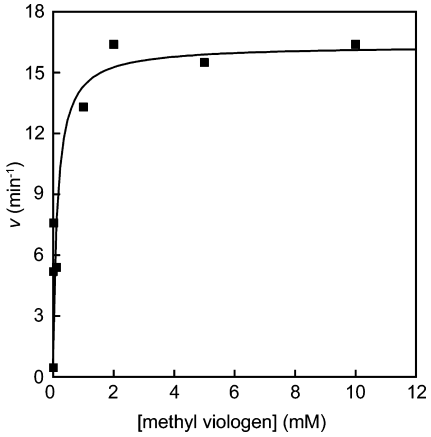

Figure 3. *Bs*QueE activity is enhanced by increasing methyl viologen concentration when electrons are supplied by dithionite. The data were fit using eq 2 to determine k_{CDG}. The activity of *Bs*QueE was 0.2 min^{-1} in the absence of methyl viologen.

conducted similar measurements with small molecule redox mediators with midpoint potentials ranging from −0.446 to −0.276 V. None of the mediators supported *Bs*QueE turnover in the absence of dithionite. However, as shown in Figure 4, at saturating concentrations of each mediator, we observe activities that are comparable to those observed with *Bs*Fld homologues and dithionite. Therefore, the differences in midpoint potentials of Fld homologues are not the source of the differential activation of *Bs*QueE by these proteins.

Uncoupled Cleavage of SAM versus Activity. All members of the radical SAM superfamily catalyze the reductive cleavage of SAM to afford a radical species, usually dAdo•, to initiate catalysis. However, it has been well-documented that these enzymes can reductively cleave SAM in a manner uncoupled from catalysis in the absence of substrate.[27−31] While this activity is biologically undesirable, it has proven to be a very useful activity for characterizing an enzyme as a member of the radical SAM superfamily in the absence of substantial quantities of substrate or in the event the substrate has yet to be determined.[28−30] However, there is little direct comparison of the propensity for uncoupled cleavage relative to product formation using various reductants.

The differences in *Bs*QueE activity that are achieved by dithionte alone relative to those observed in the presence of Fld may reflect a high proportion of abortive cleavage of SAM that masks the true activity. To address this, we measured the rate of formation for CDG and 5′-deoxyadenosine (dAdo) with dithionite alone or in the presence of the Fld homologues

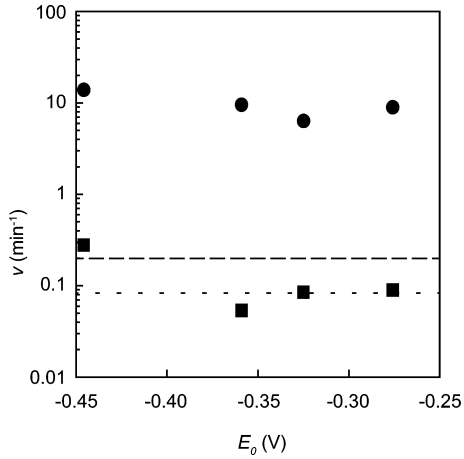

Figure 4. Velocity of CDG formation (●) or dAdo formation (■) as a function of the reduction potential of the redox mediator used in the activity assay. Dithionite was the reductant used in all of the reactions. The redox mediators used were methyl viologen (E_m = −0.446 V),[20] benzyl viologen (E_m = −0.359 V),[20] neutral red (E_m = −0.325 V),[21] and lissamine blue BF (E_m = −0.276 V, at pH 7.5).[22] The dashed lines represent the rates of formation for CDG (−−−) and dAdo (---) (0.2 and 0.08 min^{-1}, respectively) when *Bs*QueE was activated by dithionite alone.

and with Fpr/NADPH in place of dithionite (Table 1). dAdo was readily detectable (0.08 min^{-1}) when dithionite was used to deliver the reducing equivalents. In the presence of dithionite, the ratio of the rate of turnover to abortive cleavage is 2.5, which increases only slightly to 6.4 when *Ec*FldA is added to mediate the reduction. In the presence of *Bs*YkuN or *Bs*YkuP, however, 10-fold more turnover to reductive cleavage was observed relative to that with *Ec*FldA. The increase appears to arise largely from the ability of Fld homologues to support turnover, as the k_{dAdo} values measured with *Ec*FldA, *Bs*YkuN, and *Bs*YkuP with dithionite as the reductant are within experimental error of one another. It is noteworthy that dAdo does not accumulate to detectable quantities when NADPH supplies the reducing equivalents through Fpr/Fld, indicating that the reductive cleavage of SAM to form dAdo• is tightly coupled to turnover.

The experiments measuring k_{CDG}/k_{dAdo} could not eliminate the possibility that differences in the midpoint potentials of Fld homologues are responsible. Therefore, we examined the ratio of turnover to abortive cleavage when redox mediators replaced the flavodoxin homologues (Figure 4). Dithionite served as the reductant in these experiments. We note that in the presence of some redox mediators, quantifying the dAdo concentration was difficult because so little dAdo was being produced. In cases in which we could measure detectable dAdo, the rate of formation was only slightly greater than that measured in the presence of dithionite alone. Overall, there appears to be no or perhaps a weak correlation between the midpoint potential of the mediator and the k_{CDG}/k_{dAdo} ratio. We conclude from these observations that the relative differences between the Fld systems may be, in fact, inherent to their interactions.

■ DISCUSSION

Reductive activation is essential for the function of the >65000 members of the radical SAM superfamily, which are distributed in 5136 species (Pfam PF04055 family).[32] Of the three classes that comprise the superfamily, two use SAM as a cosubstrate

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whereas the class I enzymes re-form SAM on each turnover.[33] Despite the central role that reductive cleavage plays in the function of all these enzymes, to date, there have been very few systematic studies of this reaction or the possible *in vivo* pathway for activation of these enzymes.

Flavodoxins are small FMN-dependent proteins found in bacteria and algae that mediate electron transfer events *in vivo*. A role for flavodoxins as nonspecific mediators of reducing equivalents is suggested by the ability of flavodoxin from one species to interact with diverse targets. For example, *Ec*FldA interacts with flavodoxin reductase, cobalamin-dependent methionine synthase, and the radical SAM enzymes biotin synthase, pyruvate formate-lyase activating enzyme, and anaerobic ribonucleotide reductase.[4,6−10] Moreover, *Ec*FldA is commonly used *in vitro* to activate radical SAM enzymes from diverse bacterial sources.[11−13,15,17,18]

Many bacterial genomes encode two flavodoxin homologues. In *E. coli*, only one of the homologues, *Ec*FldA, is absolutely essential for cellular viability, suggesting that these proteins have nonoverlapping functions *in vivo*.[34] The *B. subtilis* genome also encodes two flavodoxin homologues (*Bs*YkuN and *Bs*YkuP), which in addition to its upstream redox partner, flavodoxin reductase, support turnover of the P450 enzyme BioI.[23] In this paper, we have shown that the two *B. subtilis* flavodoxins, as well as FldA from *E. coli*, support turnover of the radical SAM enzyme QueE from *B. subtilis*. Interestingly, both *Bs*YkuN and *Bs*YkuP are more efficient activators of *Bs*QueE than *Ec*FldA, with *Bs*YkuN and *Bs*YkuP both supporting *Bs*QueE activity at the same level under saturating conditions. However, the efficiency of the activation as measured by k_{CDG}/K_{FLD} indicates that *Bs*YkuN is preferred by nearly 10-fold. The *in vivo* relevance of this observed preferential activation is unclear because the cellular concentrations and expression profiles of *Bs*YkuN, *Bs*YkuP, and *Bs*QueE are currently unknown.

The dAdo• produced from reductive cleavage of SAM is sometimes quenched to form an abortive cleavage product in radical SAM enzymes. It has been suggested that higher proportions of dAdo from these abortive cleavage reactions are possible when dithionite is the reductant.[31] Indeed, we do not detect dAdo when *Bs*QueE is reduced with any Fld homologue using Fpr/NADPH to supply the reducing equivalents. Interestingly, with *Bs*YkuN and *Bs*YkuP, we observe substantially more CDG than dAdo even when dithionite is used. This arises almost entirely from the ∼10-fold higher activity (k_{CDG}) relative to that achieved by *Ec*FldA.

The X-ray crystal structure of QueE from *B. subtilis* is not known, but the structure of the homologue from *B. multivorans* shows that the CX_3CX_2C motif characteristic of the radical SAM superfamily is punctuated by an 11-residue insertion between the first and second Cys residues in the conserved motif.[18] The additional residues form a 3_{10}-helix at the surface of the protein, further burying and shielding the [4Fe-4S] cluster from the solvent. In contrast to the *B. subtilis* QueE, dithionite appeared to more efficiently support turnover by the *B. multivorans* homologue relative to that of *Ec*FldA.[18] Whether robust activation of *B. multivorans* QueE by the cognate Fld homologue will be the same as that observed here with the *B. subtilis* QueE/Fld system remains to be established. On the basis of the observations presented here, we predict that protein sequence/surface specific interactions will play a significant role in Fld-dependent activation of radical SAM enzymes and that a better understanding of how the species specific Fld/radical SAM partners interact will be essential in studies of the enzymes in the radical SAM superfamily.

■ ASSOCIATED CONTENT

Supporting Information

Primers used to clone the three flavodoxin homologues used in this study from their respective genomic DNA. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00210.

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Author Contributions

A.P.Y. cloned and purified the *E. coli* FldA used in these experiments. N.A.B. and V.B. designed and conducted the experiments and wrote the manuscript.

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Notes

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■ ABBREVIATIONS

SAM, *S*-adenosyl-L-methionine; dAdo•, 5′-deoxyadenosyl radical; dAdo, 5′-deoxyadenosine; NADPH, reduced β-nicotinamide adenine dinucleotide phosphate; $NADP^+$, β-nicotinamide adenine dinucleotide phosphate; FMN, riboflavin 5′-monophosphate; Fld, flavodoxin; CPH_4, 6-carboxy-5,6,7,8-tetrahydropterin; CDG, 7-carboxy-7-deazaguanine; DEAE, diethylaminoethyl; FldA, *E. coli* flavodoxin; Fpr, *E. coli* ferredoxin(flavodoxin):$NADP^+$ oxidoreductase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; IPTG, isopropyl β-D-1-thiogalactopyranoside; PIPES, 1,4-piperazinediethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; QueE, CDG synthase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; Tris, tris-(hydroxymethyl)aminomethane; LB, Lennox broth.

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